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Alteration of intersubunit acid–base pair interactions at the quasi-threefold axis of symmetry of *Cucumber mosaic virus* disrupts aphid vector transmission

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ABSTRACT

In the atomic model of Cucumber mosaic virus (CMV), six amino acid residues form stabilizing salt bridges between subunits of the asymmetric unit at the quasi-threefold axis of symmetry. To evaluate the effects of these positions on virion stability and aphid vector transmissibility, six charged amino acid residues were individually mutated to alanine. All of the six engineered viruses were viable and exhibited near wild type levels of virion stability in the presence of urea. Aphid vector transmissibility was nearly or completely eliminated in the case of four of the mutants; two mutants demonstrated intermediate aphid transmissibility. For the majority of the engineered mutants, second-site mutations were observed following aphid transmission and/or mechanical passaging, and one restored transmission rates to that of the wild type. CMV capsids tolerate disruption of acid–base pairing interactions at the quasi-threefold axis of symmetry, but these interactions are essential for maintaining aphid vector transmissibility.

Introduction

Cucumber mosaic virus (CMV) is one of the most geographically widespread plant virus species. Three properties responsible for its global distribution are its exceptionally wide host range, its movement in and transmission via seed, and its effective transmission by many different species of aphids (Palukaitis and Garcia-Arenal, 2003a, 2003b). Most plant viruses have evolved relationships with vectors to facilitate their transmission, including insects, nematodes, mites, fungi, and protist vectors. Among these, insects are the most important and aphids, in particular, are the most common of the vectors. The relationship between viruses and insect vectors are characterized with regard to whether the virus crosses membrane barriers and circulates within the insect (circulative vs. non-circulative), with regard to whether the virus replicates in the insect (propagative versus non-propagative), and temporally with regard to the time required for efficient acquisition and delivery of virus (persistent and nonpersistent) (Nault, 1997).

CMV is transmitted by aphid vectors in a non-circulative, nonpersistent manner (Ng and Perry, 2004). The relationship between the CMV and the vector is transient. The insect can acquire virions from, or transmit them to, a plant in seconds to minutes, and the virus does not cross membrane boundaries or circulate in the aphid. The transmission properties of the CMV and related members of the genus *Cucumovirus* are determined solely by the capsid protein (Chen and Francki, 1990; Gera et al., 1979). This stands in contrast to other nonpersistently, aphid transmitted viruses for which a nonstructural, viral-encoded 'helper component' is required in concert with the capsid protein to mediate aphid transmission. In both scenarios, the most compelling mechanistic model involves the binding of virions directly or indirectly (via helper component) to a ligand in the mouthparts of the insect (Pirone and Perry, 2002). The aphid ligand is likely to be concentrated at the tip of the stylet, and salivation is postulated to facilitate the release of virions from bound sites during the process of feeding and the delivery of virus into plant cells (Fereres, 2007; Martin et al., 1997; Wang et al., 1996).

A virus-centric approach to better understand mechanisms of vector transmission has been to combine structural studies of virions or viral proteins with mutational analysis to identify structural domains that are determinants of transmission. A limiting factor in this approach has been the limited availability of atomic structures for the virions and proteins of interest. For example, elegant studies have identified essential regions of helper components of potyviruses and Cauliflower mosaic virus (Moreno et al., 2005; Syller, 2005), but structural information on these viruses is only available in low resolution models. Modeling studies of the structure of geminiviruses have resulted in predictions of the capsid protein structure based on cryoelectron microscopic reconstructions (Bottcher et al., 2004; Zhang et al., 2001). Complementary studies in which mutants defective in transmission contained

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mutations localized to specific regions of the capsid protein have identified domains that are likely involved in vector transmission (Caciagli et al., 2009; Hohnle et al., 2001; Kheyr-Pour et al., 2000; Noris et al., 1998; Soto et al., 2005). In members of the genus *Nepovirus* that are transmitted by nematodes, the capsid protein has been shown to be a sole determinant of both vector transmission and vector specificity (Andret-Link et al., 2004; Marmonier et al., 2010). There is an atomic model for nepoviruses (Chandrasekar and Johnson, 1998), and domains of the capsid protein involved in transmission have been identified (Schellenberger et al., 2011). In studies on the fungal transmitted *Cucumber necrosis virus* (CNV), the capsid protein has been shown to mediate the binding of virions to zoospores during the process of transmission (Kakani et al., 2001). In this system, the molecular basis for this vector transmission is thought to require dynamic changes in virion structure (Kakani et al., 2004).

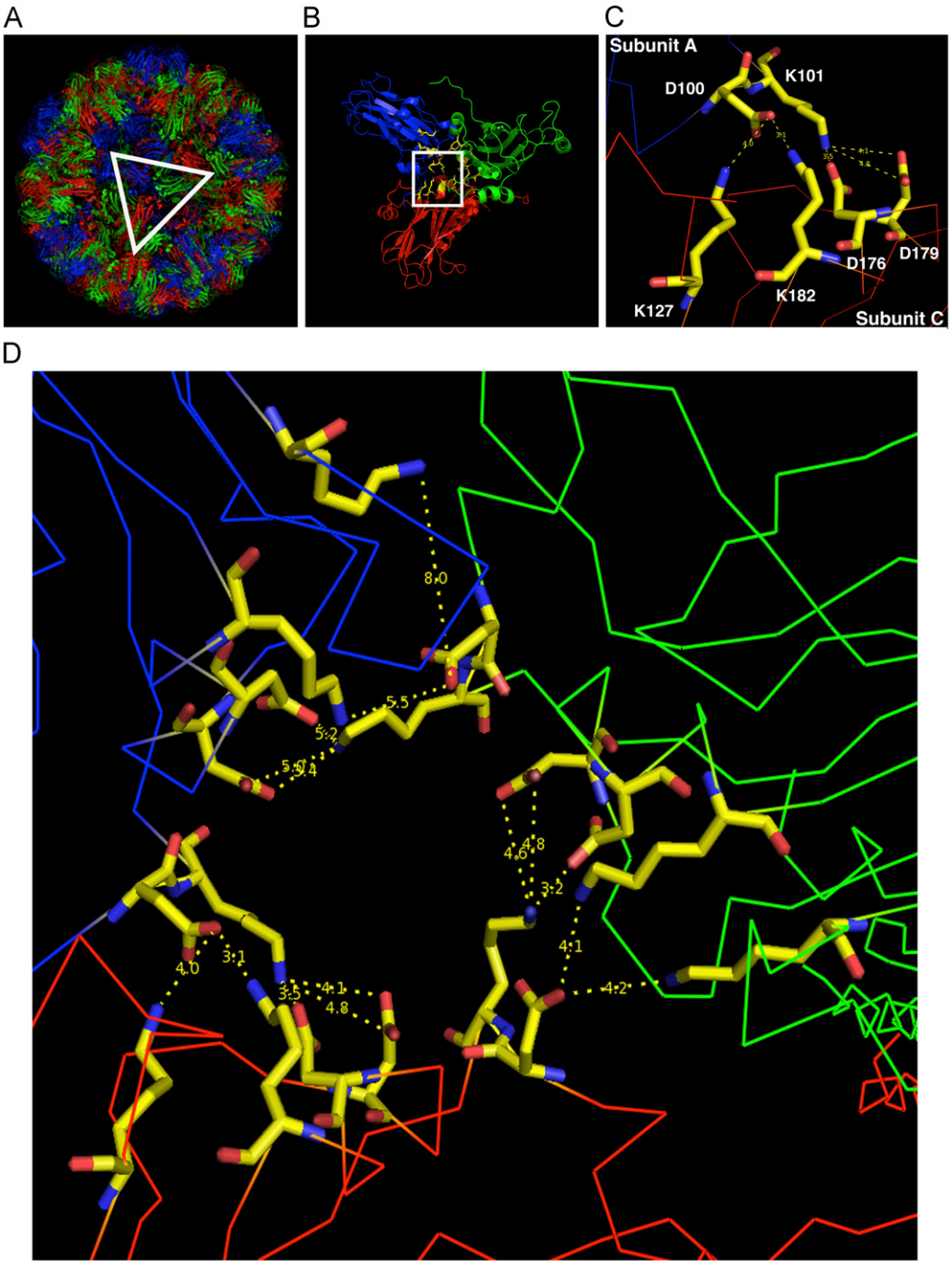

Fig. 1. Telescoping view of the quasi-threefold axes of symmetry and acid–base pair interactions in virions of *Cucumber mosaic virus* (CMV). (A) Virion of CMV with individual capsid protein subunits pictured as ribbon models and colored; subunit A is blue, subunit B is green, and subunit C is red. The triangle highlights an asymmetric unit at the quasi-threefold axes of symmetry. (B) An enlargement of the highlighted area in the previous panel, one of the 60 quasi-threefold axes of symmetry. (C) An enlargement of the interface between subunits A and C showing the amino acid side chains forming acid–base pairs with distances between pairs measured in Angstroms. (D) An enlargement of the interfacial amino acids at the quasi-threefold axis of symmetry, showing acid–base pair amino acid side chain interactions and their distances in Angstroms at the A–B, B–C, and C–A subunit interfaces.

An understanding of the biological properties of CMV has been facilitated by an atomic structure for the virus (Smith et al., 2000). Mutational analyses have shown that changes of amino acids at the surface of virions can alter aphid vector transmission (Liu et al., 2002), and changes at positions thought to be involved in subunit interactions and stability will also affect transmission (Ng et al., 2000). In this study of CMV, mutations were introduced at the positions of six charged amino acid residues involved in stabilizing salt bridges between subunits of the asymmetric unit at the quasi-threefold axis of symmetry. Each of the salt links investigated involve a single charged residue on one subunit and two on the other. Mutating either of the two single charged residues (D100 and K101) would be expected to have a greater effect than changes in the other four residues (K127, D176, D179, and K182). This is because in each case the single charged residues provide the only charge for association, while mutating any of the other four positions always leaves a complementary charge contributing to stability. Our results demonstrate that amino acid changes internal to the virion capsid are capable of disrupting aphid vector transmission while virion infectivity and stability is maintained.

Results

Acid–base pair interactions and mutational analyses at the quasi-threefold axis of symmetry

In the crystal structure of CMV, subunit interactions at the quasi-threefold axis of symmetry were observed to be stabilized by a network of salt bridges (Smith et al., 2000). These subunit interactions are located at the interfacial regions of subunits A, B, and C, and involve six subunit amino acids; D100, K101, K127, D176, D179, and K182. These amino acid side chains are located within a pore at the center of the quasi-threefold axis of symmetry (Fig. 1A, B), and represent a portion of the solvent accessible surface area of the virion cascading down this central pore (Fig. 2A, B). Three amino acids involved in acid–base pairings are located within α-helices (D100, K101, and D179), while D176 is located within a turn, directly before an α-helix, K182 is at the end of an α-helix, and K127 within a β-sheet (Smith et al., 2000). There are five possible acid–base linkages between each pair of subunits for a total of 15 possible salt bridges at each asymmetric axis (Fig. 1C, D). Quasiequivalence is demonstrated and the salt bridge distances between all subunits are comparable, with one exception. The K127 in the A subunit is oriented differently than in subunits B and C, resulting in a longer K127–D100 side chain distance, 8.0 Å in the A–B subunit interface (shown at the top of Fig. 1D) versus 4.0 and 4.2 Å at the two other interfaces; this difference was reconfirmed in the original electron density of the crystallographic model (Smith et al., 2000).

To understand the role of these acid–base pair interactions in virus viability, stability, and vector transmission, the relevant charged amino acid side chains were mutated individually to alanine. Six modifications in the CMV-Fny-Spe/Eco capsid protein gene (Mello et al., 2010) were individually engineered, resulting in the mutant constructs D100A, K101A, K127A, D176A, D179A, and K182A (Table 1). Depending on the mutated position, one, two, or three possible acid–base pair linkages were disrupted

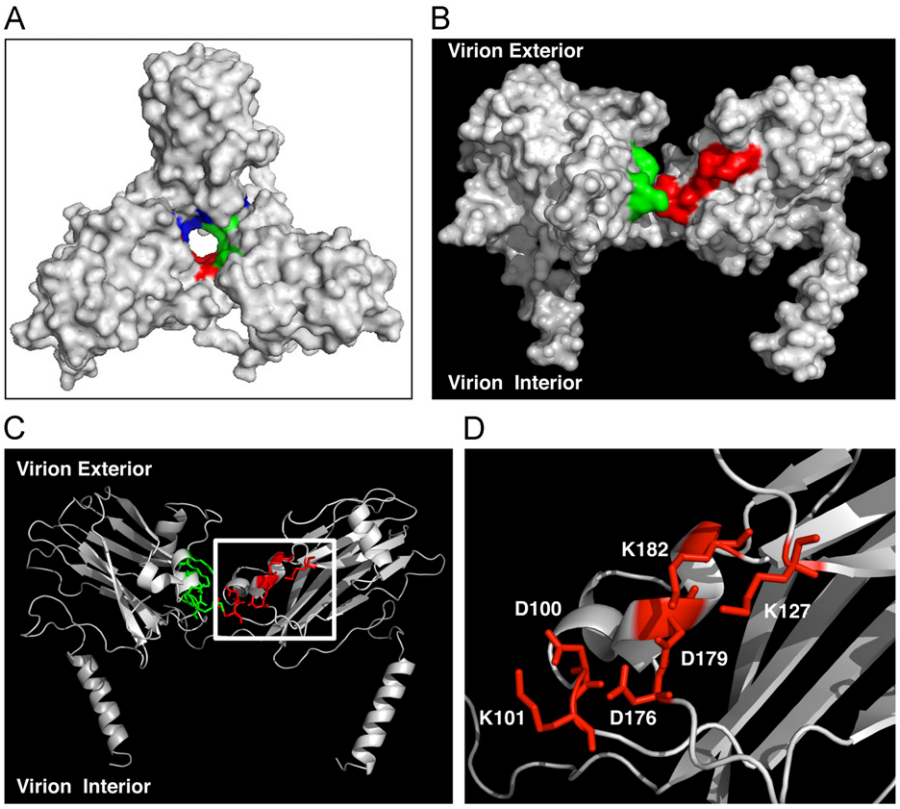

Fig. 2. Solvent accessible surface area and location of mutated amino acid side chains. (A) The solvent accessible surface (SAS) area at the quasi-threefold axis as depicted from the exterior of the virion, with amino acids involved in acid–base pairing colored according to the subunit in which they are located; subunit A is blue, subunit B is green, and subunit C is red. (B) Subunits B and C at the quasi-threefold axis visualized as the SAS and oriented with the exterior of the virion at the top of the image and the interior, RNA-proximal region, at the bottom. Subunit A is removed from the forefront of the image to facilitate visualizing the interior of the pore. The SAS of the amino acids involved in acid–base pairing are colored according to the scheme in (A). (C) The same view as panel (B), but with amino acids involved in acid–base pairing represented as sticks and colored according the scheme as described in panel (A), and the remainder of the polypeptide chains rendered as a gray ribbon. The rectangle highlights amino acids in subunit C involved in acid–base pairing. (D) An enlargement of the highlighted area in the previous panel, with relevant amino acids labeled.

Table 1
Symptoms and aphid vector transmissibility of *Cucumber mosaic virus* mutants.

Virus[a]	Symptoms			Aphid transmission[b]
	Tobacco		*C. quinoa*	
	Local	Systemic		
CMV-Fny-Spe/Eco (wild type)	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	78/80 (0.309)
Mutant D100A	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	2/34 (0.006)
Mutant K101A	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	0/35 (<0.003)
Mutant K127A	Chlorotic lesions	Mosaic, leaf deformation	Chlorotic lesions	3/25 (0.013)
Mutant D176A	Chlorotic and necrotic lesions	Mosaic, severe necrosis	Chlorotic lesions	2/30 (0.007)
Mutant D179A	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	12/25 (0.063)
Mutant R181A	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	2/20 (0.010)
Mutant K182A	Chlorotic lesions	Mosaic, stunting	Chlorotic lesions	22/25 (0.191)

[a] Mutant viruses were constructed by engineering mutations into the parental RNA 3 cDNA construct pFny309-Spe/Eco used to generate the parental virus CMV-Fny-Spe/Eco. This virus is wild-type with respect to the capsid protein gene sequence.
[b] Transmissions performed with 10 aphids (*Aphis gossypii*) per plant; numbers indicate number of plants systemically infected/total number of plants challenged. The value in parentheses indicates the estimated likelihood of transmission (frequency) by single aphids.

(Fig. 3A). An adjacent charged amino acid (R181) that had not been shown to be involved in acid–base pair interactions between subunits at the quasi-threefold axis of symmetry (Smith et al., 2000) was also mutated (R181A in Table 2, Fig. 3B).

All wild type acid–base pairs between capsid protein subunits are not essential for viability and virion formation

All six of the mutants engineered to disrupt acid–base pair interactions were viable (Table 1) and relatively stable, as virions could be purified by standard procedures involving disruption in high salt conditions (1.5 M sodium citrate) in the presence of chloroform. Virus with the wild type CMV-Fny capsid protein (CMV-Fny-Spe/Eco) produced a phenotype of local chlorotic lesions and systemic mottle-mosaic and stunting in *N. tabacum* and of necrotic local lesions without systemic infection in *C. quinoa* (Table 1). Mutants D100A, D179A, R181A and K182A all exhibited a wild type phenotype for both local and systemic symptoms in these hosts. By contrast, two mutants gave rise to altered symptoms in tobacco. Mutant D176A induced severe necrotic local and systemic symptoms in *N. tabacum*, while infection with mutant K127A resulted in a pronounced mosaic with foliar growth distortion (wrinkling and curling) (Table 1). An increase in the severity of symptoms and necrosis after engineering a point mutation in the CMV capsid protein has been observed previously (Liu et al., 2002). In all cases, the capsid protein genes of virus from systemically infected leaves were sequenced to confirm the retention of the introduced changes and the absence of additional mutations.

One mutation, K101A, initially appeared to be lethal to the virus, as in 15 transcript-inoculated plants from three-independent experiments, a systemic infection was not observed and virus could not be detected in the upper, uninoculated leaves by ELISA or PCR. By contrast, systemic infections were observed and virus detected in nearly all plants inoculated with transcripts of the other mutant viruses. Transcripts of the wild type virus were included as a positive control at the time of all inoculations and all of the inoculated plants were systemically infected (data not shown). In subsequent experiments, a total of 45 additional plants were inoculated with transcripts of mutant K101A and 10 became systemically infected, with symptoms that paralleled those of a wild type infection in *N. tabacum*. Even in the absence of a systemic infection, in two separate cases the virus could be detected in transcript inoculated leaves (as opposed to upper non-inoculated leaves), when assayed by PCR at 22–35 days after inoculation. Sequencing of the capsid protein gene from systemically infected leaves showed that the engineered mutation was retained without additional second-site mutations in eight independent infections (the other two isolates from systemic infections were not sequenced). Thus, with respect to the six amino acid positions involved in acid–base pair interactions, all could be mutated without disrupting either virion formation or the systemic infection of tobacco, although with the K101A mutation, a reduced efficiency of infection following transcript inoculation was observed.

Introduced mutations alter the charge, but not stability of virions

To test for differences in the surface charge of mutant virions, purified virus was electrophoresed in a non-denaturing agarose gel. The migration of intact virions in an agarose gel is visualized through staining of the encapsidated RNA with ethidium bromide (Ng et al., 2000). Distinct bands represent virions (uniform macromolecular structures), whereas smearing occurs when these structures are disrupted, aggregate, or are altered in their shape and charge. Fig. 4 shows the banding pattern observed when virions were electrophoresed for a relatively short time (20 min). When electrophoresis was carried out for longer periods, differences in mutant virion migration were more readily visualized, but this was accompanied by band smearing. Virus with the wild type CMV-Fny capsid protein, along with mutants D100A, D176A, D179A, and K182A, migrated as a single band. Virions of mutant K101A co-migrated with the wild type virus whereas those of mutants D100A, D176A, and D179A were slightly retarded in their migration and those of mutants K127A and K182A migrated further than the wild type. Thus, under the buffer conditions employed, the net charge of most of the mutant virions was altered relative to the wild type. For mutants K101A and K127A, an additional slower migrating band was also observed. While the nature of the second band is not understood, it was observed in five independent preparations of purified virions, and the band did not correspond in migration to that of viral RNAs (data not shown).

To assess relative stabilities of the mutant virions, a urea disruption assay was employed that has previously been used to reveal differences in the physical properties of CMV mutants. When exposed to increasing concentrations of urea, a change in the staining intensity of virions of the wild type CMV-Fny was observed between 3 M and 4 M (Fig. 5). A treatment of wild type virions was included in every gel and this change was consistently observed. A similar pattern of sensitivity to urea was observed for all the mutants tested (mutants D100A, K127A, D176A, D179A, and K182A) (Fig. 5A–E). While stained bands for mutants D100A, D176A, and D179A appeared to be completely eliminated in 4 M

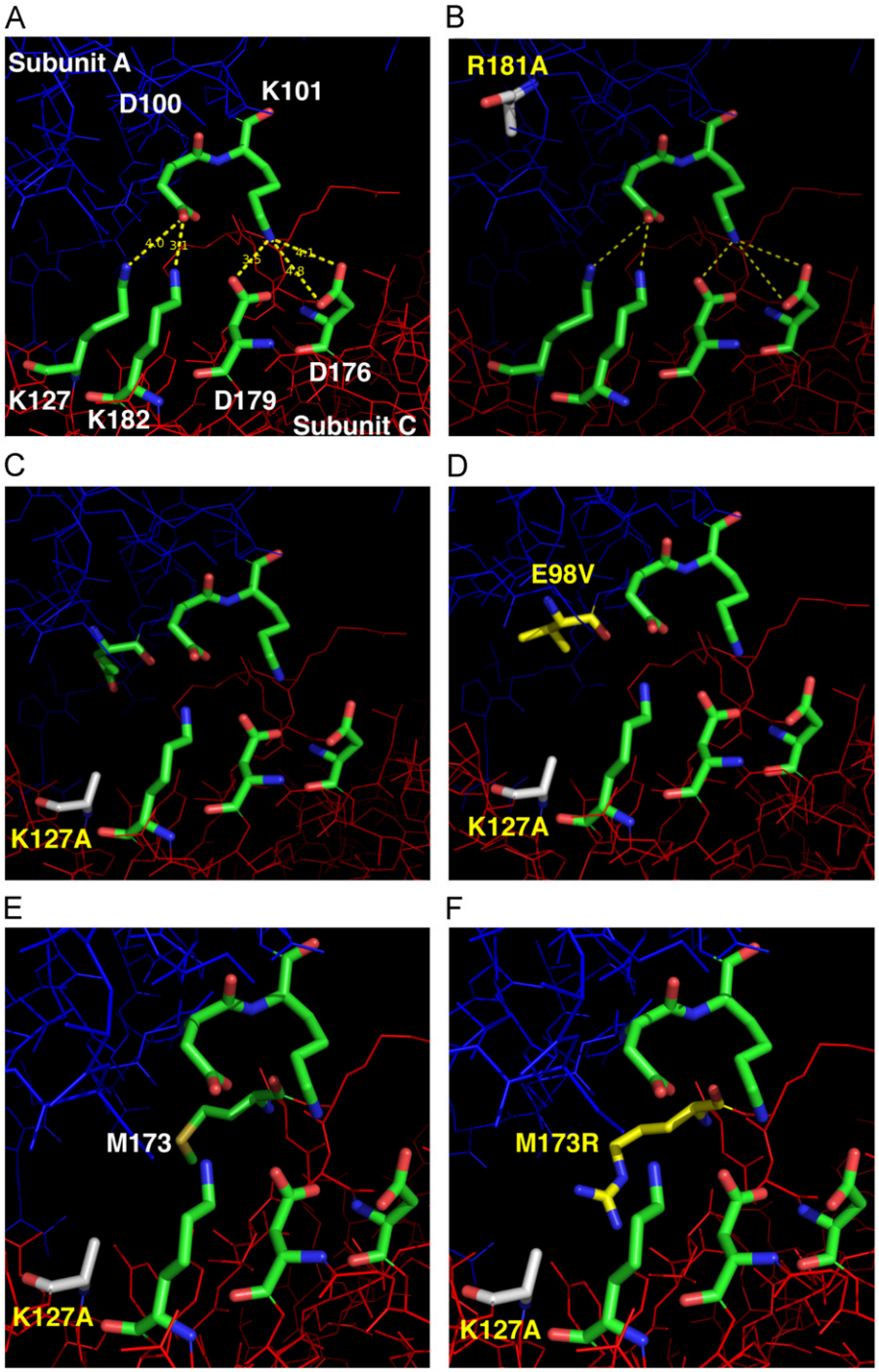

Fig. 3. Amino acids involved in acid–base pair interactions at the quasi-threefold axes and the positions of introduced and spontaneous mutations. Amino acids interacting at the quasi-threefold axis are colored according to element: carbon green, nitrogen blue, and oxygen red. The interactions depicted are those between subunits A and subunit C. (A) Wild-type CMV with distances between acid–base pairs shown in Angstroms; the side chains shown were individually mutated in the construction of described mutants. (B) Mutant R181A, an introduced mutation at a position not modeled as participating in acid–base pairing at the quasi-threefold axis of symmetry. (C–F) Second-site mutations observed following the introduction of the engineered K127A mutation and mechanical passaging. Engineered mutant amino acids and second-site mutations are colored according to the scheme noted above, except that carbons are gray or yellow, respectively. (C) Engineered mutant K127A with E98 highlighted, (D) K127A with the second-site mutation E98V, (E) engineered mutant K127A with M173 highlighted, and (F) K127A with the second-site mutation M173R. Amino acids E98 and M173 are not known to be involved in acid–base pairing, but are located at the interfacial region of the quasi-threefold axis of symmetry.

urea, bands for mutants K127A and K182A were still present, but at a decreased intensity, much like the wild type virion at this concentration. There was also a slight decrease in staining at 3 M urea for mutants D100A, D176A and D179A. In past studies, relatively unstable mutants exhibited complete disruption at urea concentrations of 1 or at 2 M (Ng et al., 2000, 2005). The secondary band in mutant K127A, which is retarded in migration compared to the capsid protein, appeared to have a gradual decrease in staining from 2 M to 4 M urea. A urea disruption assay for mutant K101A is not shown, as the phenotype of purified virions electrophoresed in an agarose gel was variable, even without exposure to urea. Preparations of the mutant K101A virions, as purified five times from separate plants, exhibited variable staining intensities in the faster migrating band that co-migrated proximal to the wild type

Table 2
Second-site mutations, reversions, and aphid vector transmissibilities of engineered *Cucumber mosaic virus* mutants following aphid transmission and mechanical passaging of the virus in tobacco.

Virus[a]	Engineered mutation[b]	Second site mutations and reversions[c]		Aphid transmissibility of mutants with second site mutations[d]
		Nucleotide change(s)	Amino acid change(s)	
CMV-Fny-Spe/Eco (Wild type)	NA	None	None	NA
Mutant D100A-MT1[e]	+	C1547T	Silent	NT
Mutant K101A-MT1[f]	+	T1553C	Silent	NT[i]
Mutant K101A-MT2[f]	+	T1551C	Y99H	NT[i]
Mutant K101A-MT3[f]	+	C1514A	Y86stop	NT[i]
Mutant K127A-MT1[e]	+	A1549T	E98V	25/25 (> 0.275) [j]
Mutant K127A-MT2[g]	+	G1548A	E98K	1/15 (0.007)
Mutant K127A-MT3[f]	+	T1774G	M173R	NT
Mutant K127A/E98V [h]	+	A1549T	E98V	13/15 (0.182)
Mutant D176A-MT1[e]	−	C1783A C1784T	A176D	NT
Mutant D179A-MT1 [g]	+	G1833A	A193T	3/5 (0.600)
Mutant K182A-MT1[e]	−	C1627T C1724T G1800A C1801A C1802G A1831C T1832G	T124I silent A182K D192A	0/5 (< 0.022)[k]

[a] Mutant viruses were engineered into the parental RNA 3 cDNA construct pFny309-Spe/Eco used to generate the parental virus CMV-Fny-Spe/Eco. This virus is wild-type with respect to the capsid protein gene sequence.

[b] The retention of the original engineered mutation is indicated with a "+". The loss of the original engineered mutation is indicated with a "−". "NA" indicates not applicable. "NT" indicates not tested. "None" indicates that no changes were observed in the DNA sequence and/or amino acid sequence when compared to wild type.

[c] Nucleotide changes are indicated as the original nucleotide, followed by the nucleotide position as in RNA3 of CMV-Fny (NCBI accession number D10538), followed by the nucleotide observed in the sequence of the second-site mutant. Amino acid changes are indicated as the original amino acid (single letter code), followed by the amino acid position in the amino acid sequence encoded by RNA3 of CMV-Fny, followed by the amino acid encoded by the sequence of the second-site mutant.

[d] Transmissions performed with 10 aphids (*Aphis gossypii*) per plant; numbers indicate number of plants systemically infected/total number of plants challenged. The value in parentheses indicates the estimated likelihood of transmission (frequency) by single aphids.

[e] This mutant was observed after serial mechanical passaging through five generations of plants.

[f] This mutant was observed in a transcript inoculated plants.

[g] This mutant was also observed in an infection established by aphid transmission.

[h] Mutant K127A E98V was engineered to reconstruct the spontaneous mutation observed in Mutant K127A-MT1. This mutant was utilized solely to determine if aphid transmissibility was maintained in a virus engineered with mutations that arose during mechanical passaging.

[i] Aphid transmissions were not carried out with these infected plants, as the second site mutations and virus were only detected in the transcript-inoculated leaves.

[j] The *p*-value indicated in parentheses was calculated assuming transmission to 24/25 plants rather than the 25/25 observed. This strategy avoids the overestimate of 100%; see Swallow (1985) for a discussion.

[k] The *p*-value indicated in parentheses was calculated assuming transmission to 1/5 plants rather than the 0/5 observed. This strategy avoids the underestimate of 0%; see Swallow (1985) for a discussion.

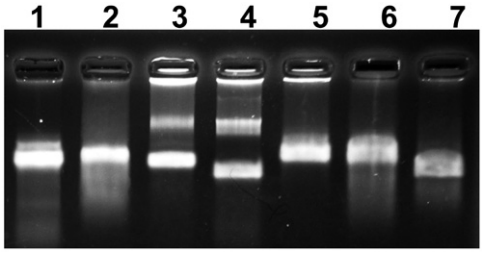

Fig. 4. Agarose gel electrophoresis of purified virions to assess differences in migration. 5 μg of each virus was electrophoresed in a 1.2% non-denaturing agarose gel for 20 min. Virions were loaded in lanes as follows; (1) wild type CMV Fny, (2) mutant D100A, (3) mutant K101A, (4) mutant K127A, (5) mutant D176A, (6) mutant D179A, and (7) mutant K182A.

and other mutant virions (data not presented). To summarize results from the disruption assay, in contrast to work with other CMV mutants (Ng et al., 2000, 2005) the five acid–base pair mutants that were tested do not radically differ from the wild type virus in their physical stabilities, as measured in this assay.

Stabilizing interactions between capsid protein subunits are required for aphid vector transmission

Of the six viruses with mutations at positions involved in acid–base pair interactions, five of the mutants exhibited altered vector transmission phenotypes (Table 1). Aphid transmissibility was nearly or entirely eliminated in four of the acid–base pair mutants, D100A, K101A, K127A, and D176A. The transmissibility of mutant D179A was intermediate with respect to the wild type virus, however it still had a significantly lower transmission rate when compared to wild type. Mutant K182A was not significantly affected in its ability to be transmitted by an aphid vector. One possible explanation for a reduction in the transmission efficiency for mutant virions was that the transmitted progeny represented aphid-transmissible revertants. To test this, plants infected via aphid transmission were used as source plants in a subsequent aphid transmission experiment. The aphid transmission frequencies in these secondary transmissions were comparable to those observed in the initial work with transcript-inoculated plants (Table 1) and there was no indication of a reversion in this phenotype (data not shown). Transmission experiments were performed using ten aphids per target plant, a number chosen to give rise to near 100% infection with virus expressing the wild type capsid protein. Aphid transmission of the wild type CMV-Fny-Spe/Eco was observed for 78/80 plants; there was 100% transmission in 14 of 16 experiments. To facilitate comparisons of the aphid transmissibility of mutants, estimates of the likelihood of transmission by single aphids were made. This is a standard methodology for group testing (Gibbs and Gower, 1960; Perry and Francki, 1992; Swallow, 1985) and has been empirically validated (Gildow et al., 2008). Virus with the wild type capsid protein (CMV-Fny-Spe/Eco) exhibited an estimated transmission frequency by single aphids of 0.309. This compares with values of 0.003–0.013 for the four defective mutants, a reduction of more

than 20-fold (Table 1). Mutant D179A showed a significantly reduced but intermediate estimated single aphid transmission frequency of 0.063 (Table 1; $p < 0.05$). Mutant K182A was transmitted by aphids at a rate more comparable to that of the wild type virus with an estimated single aphid transmission frequency of 0.191. The mutant R181A was constructed because this charged amino acid position is proximal to the quasi-threefold axis, but not observed to be involved in acid–base paired interactions in the crystallographic model. A mutation at this position also resulted in the near elimination of aphid transmissibility with an estimated single aphid transmission frequency of 0.010. Thus, while one can conclude that altering most amino acid positions with salt bridge contacts at the subunit interface can radically alter vector transmission, other charged amino acids in this region can affect transmission.

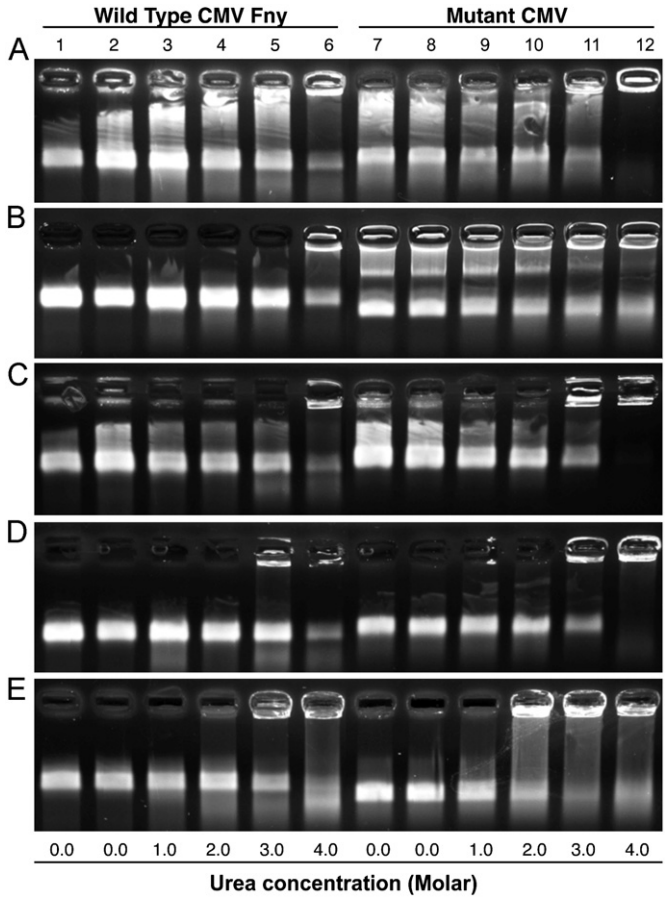

Fig. 5. Urea disruption assay of wild type and mutant purified *Cucumber mosaic virus* virions. All virions were purified by standard CMV purification procedures followed by exposure to increasing concentrations of urea (0–4 M) in 1X TAE. Virions were subjected to 1.2% TAE non-denaturing agarose gel at pH 9 and 4 °C, followed by staining with ethidium bromide. All mutant virions were run under parallel conditions with wild type virions. Lanes 1–6 represent wild type CMV exposed to increasing concentrations of urea (0–4 M urea) in all agarose gels and lanes 7–12 represent mutants (A) D100A, (B) K127A, (C) D176A, (D) D179A, and (E) K182A.

The virus titers of wild type and mutant viruses are comparable

One explanation for an observed alteration in aphid vector transmissibility could be a reduction in the concentration of virus in leaves probed by the vector. To address this, the concentration of wild type and mutant viruses was measured in plants at 12, 18, and 22 days post-transcript inoculation. There were modest differences in titers between timepoints, but with two exceptions, none of the mutants deviated statistically from the wild type concentration of virus ($p < 0.05$) at any of the three timepoints (Fig. 6). At day 12, the titer of the D179A mutant was higher than the wild type virus, and at day 18, the titer of the D176A mutant was lower than the wild type virus. At days 18 and 22, only one of the three mutant K127A samples could be included in the analysis, as the remaining two plants harbored virus with second site mutations. Titer analysis of K101A was not performed because plants that were inoculated with transcripts did not develop symptoms until 22–23 days post-inoculation, and it was difficult to reproducibly obtain systemically infected plants. While data for the K101A mutant was not obtained in the viral titer experiment, five independent preparations of mutant K101A were made 22 days post-inoculation, and the yields of this mutant (0.236 mg virus per gram plant tissue, standard error of 0.004) were about half that of the wild type virus at 12 days post-inoculation (0.563 mg virus per gram plant tissue, standard error of 0.107; Fig. 6). The timepoints of 22 and 12 days post inoculation were chosen for comparison, because they most closely mimicked timepoints at which aphid transmissions were performed and represented viral titers at the onset of prominent systemic symptoms. In summary, for five of the six mutants, the single amino acid mutations to alanine, did not alter the virus ability to accumulate in tobacco.

Selection for second-site mutations

During the course of these studies, the capsid protein gene sequence was determined for virus in transcript-inoculated source

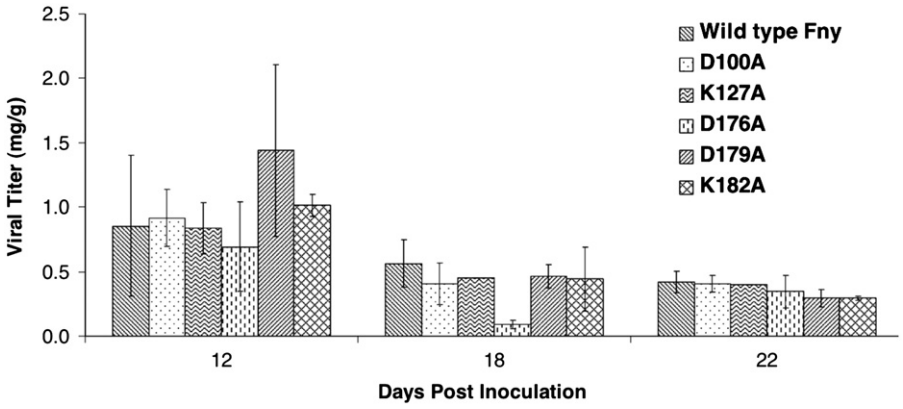

Fig. 6. Viral titers measured 12, 18, and 22 days after inoculation of tobacco with RNA transcripts. Virus was purified from single leaves, with three preparations from three separate plants for each virus at each time point. For the K127A mutant, data are for one plant at the 18 and 22 day time points, as only one of the three infected plants maintained K127A with no second site mutation.

plants and in plants infected following vector transmission. For all of the experiments described above, the introduced mutations were confirmed as being retained and the absence of additional spurious mutations confirmed. In 41 independently inoculated, wild type infected plants, no spurious changes were observed when the capsid protein gene was sequenced. In transcript-inoculated plants, and when viruses were propagated by mechanical inoculation, one or more second-site mutations were sometimes observed (Table 2). Additional mutations were detected in nine plants, three of which were infected by mutant K101A and three infected by mutant K127A.

Changes associated with mutant K127A were of particular interest, because the second-site mutations encoded changes of E98 and M173, positions that mapped in the atomic model proximal to position 127 at the quasi-threefold axis of symmetry (Fig. 3C, D). Two of the changes associated with mutant K101A were silent mutations and all three changes were only observed in the transcript inoculated leaves of plants that did not become systemically infected; no second-site mutations were observed in plants systemically infected with mutant K101A. Of the remaining second-site mutants, two of the infected plants harbored second-site mutations that encoded changes in positions that mapped to the βH-βI loop; these were D192A and A193T. The D192A change had previously been shown to eliminate aphid transmissibility (Liu et al., 2002). The A193T change was observed for the mutant D179A in a plant infected via aphid transmission; no second-site mutations were observed in four other plants infected with the mutant D179A via aphid transmission. Overall, one or more second-site mutations were observed for the majority of the engineered mutants. While in most plants infected with the mutant viruses the engineered mutations were retained, one engineered mutation was lost; the mutant D176A reverted (Table 2). There were no changes observed in the wild type capsid protein gene in virus derived from transcript inoculated plants and after five generations of mechanical passaging. Thus, it appears that introducing mutations encoding single amino acid changes alters the genetic stability of the virus and there is selection for second-site mutants.

Discussion

Virions of CMV are stabilized through a network of protein–protein interactions, with protein–RNA interactions being essential for virion formation. In this study, the importance of capsid intersubunit acid–base pairing at the quasi-threefold axis of symmetry of CMV was assessed by individually mutating the six participating amino acid subunits. It was observed that all six engineered mutants systemically infected tobacco and that the five tested mutants had comparable virion stabilities. Although none of the side chains involved in acid–base pair interactions were individually essential for virion formation, the engineered mutants were genetically unstable. Second-site mutations were frequently observed for most mutants after inoculation with transcripts, following aphid transmission experiments, and/or mechanical passaging in *N. tabacum*. While it was predicted that changes at positions D100 or K101 might have the greatest effects since these single positions provide the only charge for association (relative to any of the other four positions wherein mutation would still leave a complementary charge contributing to stability), this did not appear to be the case.

In relation to the outer radius, amino acid side chains mutated in this study are located about one-third to one-half of the thickness of the capsid protein inward from the outer surface (Fig. 2C D). The outer radius of the virion's protein shell measures approximately 151 Å, while the inner radius measures 86 Å, resulting in an estimated capsid protein thickness of 65 Å (VIPERdb) (Carrillo-Tripp et al., 2009). While amino acids involved in acid–base pairing at the quasi-threefold axis are distal to the virion surface, they still represent part of the solvent accessible surface area of the virion, and virion migration patterns in non-denaturing agarose gels were altered. With the exception of K101A, shifts in migration were as might be predicted. Virions of aspartic acid mutants migrated more slowly and lysine mutant virions migrated ahead of virions of the wild type virus, consistent with predicted reduced and greater electronegative charges, respectively. The migration of mutant K101A was not altered. The position of K101 is the most distal from the surface of the virion relative to the other side chains involved in acid–base pairing (Fig. 2D), being located approximately half-way between the outer- and inner-radii, and the absence of an effect on migration suggests its charge may be otherwise shielded. Virion preparations of both mutants K127A and K101A exhibited secondary bands migrating slower than the main banding region for all viruses, although the banding pattern for mutant K101A was variable. For one preparation of mutant K101A virions incubated at 16 °C for one hour in the presence of 1X TAE, the slower migrating band disappeared after incubation (data not shown). This suggested that whatever the nature of the slower migrating macromolecular complex, it was labile or its formation reversible.

All engineered mutants, with the exception of K182A, exhibited a reduction in their ability to be transmitted by an aphid vector. This altered transmission phenotype is of interest, as these amino acid positions are not proximal to the surface of the virion, rather they are located within a pore at the quasi-threefold axis. In past mutational analyses, vector transmission was also disrupted, but the engineered changes were in loop structures at the virion surface ((Liu et al., 2002); unpublished data). A working hypothesis on the mechanism of transmission is that a ligand in the mouthparts of an aphid vector binds virions, and that this binding is mediated by a charged virion structural motif consisting in part of the βH–βI loop (Liu et al., 2002). Spontaneous mutants with amino acid changes at positions within virions at subunit interfaces have previously been shown to disrupt transmission, but the defects were thought to be due to a demonstrated reduction in virion stability (Ng et al., 2000, 2005). Thus, the transmission defective mutants described in this study differ from mutants described in past studies in that subunit interactions are affected, but without grossly affecting virion stability. Although it is possible that the assembled virions of mutants are less infectious than wild type virions, we did not note any obvious difference in infectivity of either macerated leaves or purified preparations of virions operationally diluted 1:10 in resuspension buffer. In earlier studies of CMV mutants with reduced stability, purification required lower salt buffers in the absence of chloroform, but once purified, virions exhibited infectivity comparable to the wild type (Ng et al., 2000, 2005). By contrast, virions of the mutants in this study could be purified with high salt and organic solvent. We examined negatively stained particles of three of the mutants (D176A, D179A, and D100A) by transmission electron microscopy and did not observe differences in size or shape relative to the wild type virus (data not shown).

Of the observed second-site mutations, the K127A/E98V double mutation in mutant K127A-MT1 was of particular interest, because this was a compensatory mutation that restored aphid transmissibility (Table 2). This is remarkable in that although the forces of selection for fitness take place in the host plant (presumably acting on some combination of replication, cell-to-cell movement, and systemic movement), the resulting double mutant was also more fit for vector transmission. Mutant K127A proved to be genetically unstable, as two additional second-site mutants were recovered with changes E98K or M173R. In these

three cases, the side chains of the second site amino acids are located within the quasi-threefold axis of symmetry in proximity to K127A. Thus, while the original mutation was not lethal, there was selection pressure to compensate, presumably by restoring a structural or acid–base balance at the quasi-threefold axis. Other mutants were also genetically unstable, with second site mutations being observed (Table 2). This contrasts with observations on the wild type capsid protein gene, wherein sequencing was performed in multiple experiments throughout the course of this work and no additional mutations were observed.

Three models of vector transmission are compatible with a ligand binding hypothesis and the present data. It is possible that a ligand within the stylet of the aphid can interact with the virion, resulting in a structural transition necessary for successful transmission of the virus. In this model, the mutations described in this study would be expected to interfere with structural transitions essential for vector transmission. Although one can draw upon examples of ligand binding from animal virus systems such as the binding of ICAM-1 to the human rhinovirus at the canyon located near the fivefold axis of symmetry (Hogle, 1993; Xing et al., 2003), the fate of the bound animal virus (uptake) is fundamentally different from the reversible binding of nonpersistently transmitted plant viruses.

An alternative model would hold that disrupting the acid–base pair interactions within the quasi-threefold axis of symmetry disrupts the conformation of the βB–βC, βD–βE, and βH–βI loops exposed at the surface. While amino acid positions within the βH–βI loop are critical to the successful aphid vector transmission of CMV, it is not currently known how the conformation of the three loops affects transmission (Liu et al., 2002). It is possible that a specific conformation of these three loops is necessary for the successful binding and/or release of the virion from the aphid stylet. As amino acids critical for transmission lie within and at opposite ends of the βH–βI loop and at the termini of different β-strands (Liu et al., 2002), it is a formal possibility that disrupting these acid–base interactions could alter the surface structure. It should be noted that the K182A mutation, which is transmitted by the aphid vector at a near-wild type level, and D179A, which was transmitted at an intermediate level, are predicted to be flanked by salt bridges (D100-K127 and K101-D176; Fig. 3A). These flanking interactions appear to be important to and play a critical role in maintaining aphid transmissibility and presumably maintaining virion structure. Disrupting these stabilizing interactions could be causing structural changes within subunits that alter surface loop orientation and vector transmission.

A third model is that the mutations affecting subunit interactions could influence transmission by altering the dynamic properties of virions that in turn influence the binding and/or release of virions. Some virion 'breathing' or flexibility (Johnson, 2003; Reisdorph et al., 2003) is likely essential both within the host plant and for vector transmission. A plant virus model in which virion flexibility is hypothesized as necessary for vector transmission is CNV. This virus was observed to undergo conformational changes in order to bind its zoospore vector reversibly (Kakani et al., 2004). This model holds that CNV requires a swollen intermediate capable of binding its vector that then allows for transmission and plant infection. CMV is not thought to be able to undergo large-scale quaternary structure changes as for some other members of the *Bromoviridae* (e.g. *Cowpea chlorotic mottle virus*; reviewed in Johnson (2003) and Witz and Brown (2001)) and the *Tombusviridae* (e.g. CNV). For CMV, more modest dynamic properties may be required for vector transmission and this model would be consistent with the observation that: (i) amino acids involved in intersubunit interactions and distal from virion surface can affect aphid transmission, and (ii) a compensatory second site mutation at the subunit interface can restore vector transmission. Given the position of the mutated amino acids distal from the surface and the fact that the physical stability of the mutants did not appear to be altered, we propose that dynamic properties of the virion involving flexibility but not swelling are responsible for altering aphid transmission, presumably by affecting some aspect of virion binding and/or release in the stylet of the aphid vector.

In conclusion, while acid–base pair interactions located within the quasi-threefold axis of symmetry are not required for capsid protein stability or virion viability, the majority of these interactions are required for the maintenance of viral transmission by its aphid vector. We have shown that introducing single mutations of these interacting side chains, which are solvent accessible, yet not present on the most exterior surfaces of the virion, play a critical role in the transmission properties of the virions. Thus, this study presents an example of a transmission system in which amino acids distal to the surface of the virion are capable of disrupting transmission by an insect vector.

Materials and methods

Engineering Mutations into the capsid protein of CMV

PCR-based site-directed mutagenesis was utilized to introduce nucleotide changes into the capsid protein gene within pFny309-Spe/Eco, an RNA3 cDNA construct derived from pFny309 (Rizzo and Palukaitis, 1990). pFny309-Spe/Eco only differs from the parental construct in having *Spe*I and *Eco*RV restriction enzyme sites engineered to precisely flank the capsid protein coding region. As the capsid protein coding sequences in this construct are those of the wild type isolate, virus derived from pFny309-Spe/Eco is subsequently referred to as 'wild type'. To generate the mutant PCR fragments, a method developed by Higuchi et al. (1988) was utilized. The high-fidelity, thermostable polymerase Pfu (Stratagene, La Jolla, CA) was used in accordance with the manufacturer's instructions. Information regarding capsid protein structure and amino acid locations were obtained from an atomic structure of the virus (Smith et al., 2000).

To introduce mutations into the asymmetric unit of the virion, PCR fragments coding for the desired amino acid change were digested with *Pst*I and *Sal*I and ligated into a pFny309 Spe/Eco vector, that had been previously digested with *Pst*I and *Sal*I, using T4 DNA ligase (New England Biolabs, Ipswich, MA). Cloning was completed in *E. coli* ElectroMax DH5α Electrocompentent Cells (Life Technologies, Grand Island, NY).

Primers used to introduce desired mutations into the pFny309-Spe/Eco plasmid were as follows from 5′ to 3′, with mutation sites in bold and the nucleotide positions, as numbered for CMV-Fny RNA3 (accession number D10538), flanking the sequence: for mutant D100A, 1542-G**T**CACTGAATATG**CC**AAGAAGCTTGTT-1568; for mutant K101A, 1545-ACGGAATATGAT**GC**GAAGCTTGTTTCG-1571; for mutant K127A, 1623-GTGAC-AGTCCGT**GC**AGTTCCTGCCTCC-1649; for mutant D176A, 1770-GCGATGCGCGCTG**CC**ATAGGTGACATG-1796; for mutant D179A, 1779-GCTGATATAGGTG**C**CATGAGAAAGTAC-1805; and for mutant K182A, 1788-GGTGACATGAGA**GCC**TACGCCGTCCTC-1814; and for mutant R181A1788-GGTGACATG**GC**AAAGTACGCC-1808. It should be noted that base 1547 in the D100A primer is a marker coding for a silent mutation of G–T.

These forward primers were used in combination with a 3′-end primer with the sequence 5-GCATGC**CTGCAG***TGGTCTCCT*-3′, which binds to the last 9 nucleotides (italicized, positions 2216 to 2208) of RNA3 and introduces a *Pst*I restriction site (Liu et al., 2002). The restriction site in this and other primers are underlined. To produce the second PCR fragment, in accordance with the Higuchi procedure, primers complementary to each of those encoding mutations were

constructed. These complementary primers were used in combination with a forward primer overlapping the *Sal*I site in the cDNA for CMV RNA3, 1288-GTAACC**GTCGAC**GTCGTCCGC-1309.

Following the construction of the mutants, the plasmid containing the mutation was sequenced to verify the presence of the desired mutation and the absence of any mutations that would alter any wild type amino acids in the sequence. Mutants were named as follows; single letter abbreviation coding for the wild type amino acid, followed by the amino acid position at which the mutation was engineered as numbered for CMV-Fny RNA3, followed by the single letter abbreviation coding for the mutant amino acid.

Transcription of viral RNA, inoculation of mutants, and aphid transmission

In vitro transcription of linearized pFny109 (RNA1 cDNA), pFny209 (RNA2 cDNA), and pFny309 (RNA3 cDNA) and derivatives was carried out using the mMessage mMachine T7 transcription kit (Life Technologies). The three transcripts were then mixed together in equal volumes and mechanically inoculated onto tobacco (*Nicotiana tabacum* cv. Xanthi NN) and *Chenopodium quinoa*. Aphid transmissions with *Aphis gossypii* were completed 11 to 22 days post-inoculation. Aphids used for transmission experiments were reared on cucumber plants, starved for two hours at room temperature, and transferred onto symptomatic leaves (source leaves). Aphids were allowed to probe for one to ten minutes and then transferred onto healthy tobacco plants. Only aphids presumed to be feeding at the time of the experiment were used in transmissions. The experimental unit was one source plant and five target plants, with ten aphids per plant. As a negative control, aphids were allowed to probe on an uninfected tobacco source plant prior to transfer onto healthy target plants. Seven to 14 days after transmission, plants were assessed for systemic symptoms, total RNA was extracted using an RNeasy® Plant Mini Kit (Qiagen, Valencia, CA), the RNA subjected to reverse transcription and PCR (RT-PCR), and the capsid protein gene in the resulting product sequenced.

For the mechanical passaging experiments, five tobacco plants were inoculated with transcripts of wild type CMV-Fny-Spe/Eco and each of the mutants. 7–14 days post-inoculation, a symptomatic leaf was macerated in 0.05 M sodium phosphate, pH 7.0, and five new tobacco plants were mechanically inoculated. After five generations of passaging, RNA was extracted from a single leaf, subjected to RT-PCR, and the capsid protein gene in the resulting product was sequenced. To facilitate comparisons of the transmission efficiency of mutants, the likelihood of transmission by single aphids (p^*) was estimated using a group testing method (Gibbs and Gower, 1960; Swallow, 1985). This statistical approach can provide a more reliable estimate of transmissibility (Gibbs and Gower, 1960; Gildow et al., 2008). Ten aphids were used per plant, rather than one aphid per plant, increasing the number of transmission events while minimizing the number of test plants required to obtain estimates.

The variable p is the proportion of infected plants in an experiment, and p^* the maximum likelihood estimate of p, a measure of the single aphid transmission frequency.

$q=1-p$ is proportion of uninfected plants in the population and $q^*=1-p^*$ is the estimate of q. The maximum likelihood estimator (p^*) was calculated as follows;

$p\text{-}=1-q^*=1-(1-R/N)^{1/i}$, with N representing the number of test plants used (five per experiment), i representing the number of aphids feeding on each test plant (ten aphids per plant), and R representing the number of plants that became infected after aphid transmissions.

Virion purification, electrophoresis and stability assays and assessing viral titer

Virus was purified from infected tobacco according to the methods of Lot et al. (1972). Purified virions were resuspended and stored in buffer C (5 mM sodium borate, 0.5 mM EDTA, pH 9.0). To assess for difference between virion surface charge, 5 μg of each purified mutant virion, in parallel with 5 μg of purified wild type virion, were electrophoresed on a 1.2%, non-denaturing agarose gel at room temperature for 20 min. To assess the relative stability of virions, a urea disruption assay was employed (Ng et al., 2000). Purified virions (16.6 μg) were subjected to urea concentrations from 0 to 4 M, in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA) with 50 μl reaction volumes. Virions were incubated at 16 °C for 1 h and then electrophoresed on a non-denaturing 1.2% agarose gel at 4 °C for 30 min. For all experiments, a reference control with the wild type CMV-Fny-Spe/Eco was analyzed in parallel under the same conditions as the engineered mutants.

Transcripts of wild type CMV-Fny-Spe/Eco and each of the mutants were separately inoculated onto two sets of five tobacco plants. A single symptomatic, systemically infected leaf from three plants was removed 12, 18, and 22 days post-inoculation and virus was purified. Sampled leaves were the youngest expanded leaves available at the time of sampling. The concentration and yield of purified virus was determined for each of the three time-points. A sequencing reaction was completed on RNA extracted from virions for the K127A mutant to assess for the presence of any second site mutations, as this virion gained second site mutations frequently *in vivo.*

Molecular modeling

All three-dimensional images were constructed using PyMOL by DeLano Scientific (DeLano, 2002). The coordinates of CMV (PDB ID: 1f15) as presented in the crystal structure of CMV (Smith et al., 2000) were used in combination with RCSD Protein Data Base (Berman et al., 2000) in the creation of images of the virion, asymmetric unit, and all intersubunit interactions.

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